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PRINCIPAL INVESTIGATOR: J. Wade Harper, Ph.D.

CONTRACTING ORGANIZATION: Baylor College of Medicine
Houston, Texas 77030

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J. Wade Harper, Ph.D.

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Baylor College of Medicine
Houston, Texas 77030

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Breast cancer results from inappropriate cell proliferation due to mutations that disrupt normal cell cycle control. Our studies are focused on understanding the role of the p21Cip1 protein in cancer. p21 is a member of a family of proteins, including p27 and p57, that inhibit cyclin dependent kinases (Cdks). p21 is regulated by the tumor suppressor protein p53 and is thought to contribute to p53's tumor suppressor function through its interaction with Cdks. p53 is commonly mutated in breast cancer. We have examined the expression of p21 during development and in adult tissues and have found that p21 expression is highly selective and correlates with terminal differentiation. We have also analyzed the phenotype of mice lacking p21. We have found that p21 is responsible for only a subset of p53's function. Specifically, p21 is required for G1 checkpoint function but is not required for the G2 checkpoint or apoptosis induction. Moreover, animals lacking p21 do not readily develop tumors, implying that p21 is not a tumor suppressor. Additional studies have led to a further understanding of the regulation of p21 and the identity of its targets. In addition, we have found that p21 and p57 collaborate to control lung development.

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FOREWORD

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Introduction

The rate limiting step in control of somatic cell duplication is the commitment to enter S-phase. This step occurs in the Go or G1 stage of the cell cycle depending upon the growth state of the cell in question. The process of S-phase entry is complex but, in fibroblasts, can be divided into four stages as defined by Pardee (1). These stages include exit from quiescence (dependent upon growth factors such as PDGF), entry into G1 (dependent upon e.g. EGF), progression through mid and late G1 to the restriction point (dependent upon IGF-I and protein synthesis), and assembly of factors required for S phase. The restriction point is thought to reflect a point in the cell cycle where further transitions are not dependent upon mitogenic stimuli, and has therefore received the greatest attention. Initiation of DNA replication begins 1-3 hours after passage through the restriction point, and it is thought that these two events are differentially controlled. Data from a number of systems indicate that both of these transitions are dependent upon the activities of members of the cyclin dependent kinase (Cdk) family of proteins. Although much is known about the proteins that regulate the G1/S transition, the precise biochemical mechanisms which underlie this transition are not fully understood.

Cyclin-dependent kinases and positive cell cycle control. Progression through the cell cycle is dependent upon the assembly and activation of a family of Cdks (reviewed in 2-3). To date, four Cdks (Cdk2, 3, 4 and 6) have been implicated in regulation of the G1/S transition in mammalian cells. These kinases are inactive in their monomeric form and require association with members of the cyclin family of proteins, as well as phosphorylation on a conserved threonine residue, for activation. This activating modification can be catalyzed by Cdk7/cyclin H, a Cdk activating kinase (CAK) (4). In cells released from Go, D and E type cyclins are sequentially expressed during G1 (reviewed in 2). Cyclin E is expressed transiently and is degraded early in S-phase (5,6). In some cell types, cyclin D levels remain high throughout the remainder of the cell cycle (7). D-type cyclins (D1, D2, and D3) are thought to primarily activate Cdk4 and Cdk6, while cyclin E activates Cdk2 and perhaps Cdk3 (see below). Cyclin A is synthesized during S and G2 and activates Cdk2 and Cdc2. Consistent with a positive role for D-type cyclins in cell cycle entry, these genes are growth factor inducible in certain cell types (reviewed in 2).

A number of laboratories have addressed the issue of whether cyclins are required for early cell cycle events. Injection of antibodies against cyclin E can block entry of normal diploid fibroblasts into S-phase (8,9). Similar experiments with cyclin A indicate that it functions in coordinating at least two transitions, including DNA replication and passage through G2 (9-11). In cells engineered to overexpress D or E-type cyclins, entry into S-phase is accelerated by ~2 h compared with control cells, providing additional evidence that G1 Cdks are rate limiting for certain steps in the G1/S transition (12-14).

Negative cell cycle control pathways. Much of our knowledge about negative regulation of cell cycle entry emanates from studies of two tumor suppressor genes, Rb and p53 (15-17). Mutations in these are found frequently in diverse types of human cancers, and reintroduction of wild-type genes into $p53^-$ or Rb^- tumor cells can suppress the neoplastic phenotype suggesting that loss of function of these genes contributes to tumorigenesis (18-21).

Mutations in p53 are the most common lesions observed in human malignancies, occurring in greater than 50% of all tumors (17) including those of the breast. The percentage is much higher if loss of p53 function via association with viral oncoproteins (E1B of adenovirus and E6 of papilloma virus) or amplification of the p53 binding protein MDM2 are included. p53 deficient mice are prone to the spontaneous development of a

variety of tumor types (22) Cellular responses to DNA damage such as apoptosis and the G1 checkpoint are dependent on p53. p53 also controls a spindle checkpoint and prevents genetic alterations such as gene amplification (23-28).

Rb functions to coordinate cell cycle transitions and transcriptional pathways that are required for cell cycle progression (15). The Rb protein displays two prominent features: 1) functional inactivation through various mechanisms contributes to deregulated proliferation and an inability of the cell to appropriately regulate G1 decisions, and 2) periodic alterations in its phosphorylation status that correlate with particular phases of the cell cycle. In early and mid G1, Rb is hypophosphorylated, as determined by mobility on SDS-PAGE, but as cells enter late G1 (approximately co-incident with the restriction point), Rb becomes progressively hyperphosphorylated and remains so until passage through mitosis. At mitosis, Rb is dephosphorylated, possibly due to the action of a type II protein phosphatase that has been shown to physically associate with Rb (15). In addition to Rb, there are two other prominent pocket proteins p107 and p130 which are thought to have roles in regulating G1 and S phases (15). These two proteins share some mechanistic similarities with Rb in that they are phosphoproteins and are targeted by tumor virus oncoproteins.

The finding that Rb phosphorylation correlates with cell cycle progression has led to a model whereby Rb serves a timing function, linking the cell cycle regulatory machinery with the transcriptional machinery responsible for coordinating expression of genes required for S-phase (15). This is thought to be accomplished through the interaction of Rb with particular target proteins and these interactions are thought to be disrupted temporally through Rb phosphorylation. The best understood Rb binding proteins are members of the E2F family of transcription factors that regulate expression of genes required for S-phase, although it is not clear whether E2F represents the target of Rb responsible for growth suppression (15). A large body of data implicate cyclin dependent kinases in Rb phosphorylation and inactivation of Rb's growth suppressive function. We have recently shown using a microinjection-based approach that phosphorylation of Rb by cyclin D/Cdk4 relieves Rb mediated growth suppression but cyclin E/Cdk2 and cyclin A/Cdk2 are inactive in this assay even though they phosphorylate Rb extensively on sites overlapping with those phosphorylated by Cdk4/cyclin D1 (30). We identified a single Cdk4/cyclin D1 phosphorylation site that is required for Rb inactivation in this assay.

Although G1 Cdks are likely mediators of Rb phosphorylation, it appears that other substrates are also rate limiting for DNA replication when cyclins are overexpressed. We have shown that a mutant of Rb lacking all 14 consensus Cdk phosphorylation sites is by-passed by co-expression of either cyclin E/Cdk2 or cyclin D1/Cdk4 in SAOS-2 cells (31). In this setting, high levels of cyclin/kinase activity is sufficient to activate DNA synthesis without Rb phosphorylation and activation of the E2F pathway (32).

Cyclin-dependent kinase inhibitors: mediators of negative cell cycle control. There is now clear evidence that cyclin/Cdk complexes can promote cell cycle transitions whether by Rb dependent or Rb-independent mechanisms. Acting in opposition to cyclin kinases are cyclin kinase inhibitors (CKIs). These proteins fall into two classes: the CIP/KIP class and the INK4 class (33-41). These versatile molecules have potential roles in cell cycle arrest, checkpoint function and development and are likely to cooperate with Rb, p53, and other negative regulators in maintaining the non-proliferative state throughout adult life.

At the time of submission of this grant in December 1993, we and others had just found the first mammalian Cdk inhibitors p21 (33-36) as either a Cdk binding protein or as a p53 regulated gene. At that time there were many unanswered questions regarding the role p21 in cell cycle control and cancer. What was known was that p21 could bind and inhibit a number of Cdks, that it was transcriptionally regulated by p53 overexpression, and that its

chromosomal location did not mark it as an obvious tumor suppressor. In addition, it was not known whether p53 is the only regulator of p21 expression, how p21 might be used during development, or whether loss of p21 expression contributes to cancer. Since that time, however, there has been an explosion in our understanding of the mechanisms of action and biological function of p21 (42, 43). In addition, we now know that p21 is only one member of a family of inhibitors that share structural and functional characteristics. p27KIP2 was identified as a protein associated with Cdk2/cyclin E or Cdk4/cyclin D (44, 45). Subsequently, we and others identified p57KIP2 as the third member of the CIP/KIP family in mammalian cells in a two-hybrid screen designed to identify cyclin D1 interacting proteins (46, 47). These inhibitors primarily inhibit Cdk2, Cdk4, and Cdk6 containing Cdk complexes. The second group of inhibitors, the INK4 family, are selective for Cdk4 and Cdk6. This group includes the tumor suppressor protein p16.

Our understanding of these molecules has moved forward in several ways (reviewed in 48). Knockout mice have now been created for all of the CIP/KIP and INK4 proteins and the phenotypes are emerging. In addition, we now have a good picture of how these proteins work biochemically and what proteins they interact with in vivo. Moreover, we know the structure of a cyclin A/Cdk2/p27 complex that allows an understanding of the relationships between structure and function for the CIP/KIP family. Finally, we are beginning to understand the transcriptional control pathways that dictate when and where these inhibitors are expressed.

As detailed in previous progress reports and summarized below, we have made excellent progress on the original five aims of this proposal, the major goal of which was to make and analyze p21 knockout mice. Much of our data has been summarized in detail in the first three progress reports for this grant (1995-1997). Essentially all of the major goals of the proposal have been completed (Table 1). Therefore, in this report, I will first briefly summarize our findings related to the specific aims indicated above with an emphasis on published findings and will focus the body of the report on unpublished data that extends our original goals to the area of redundancies of CKIs. We have included published papers in the appendix.

Body

Progress of Specific Aims 1-5: The tasks set forth in aims 1-3 have been completed and were published in two papers (see Table 1). In short, for Aim 1, we found that p21 expression during development correlates with the process of terminal differentiation in a subset of tissues (49). Together with our analysis of p57 expression (46), we found that this class of CKIs is differentially expressed in a number of cell types in vivo. These studies also allowed us to develop a model whereby CKIs function directly in the process of differentiation (50), a property that we subsequently identified through the analysis of p57 deficient mice. For Aims 2 and 3, in collaboration with Dr. Phil Leder, we have generated p21 deficient mice and analyzed the phenotypes of both deficient animals and of cells derived from these animals (51). The results provide evidence that p21 is not required for development nor does loss of p21 predispose mice to transformation. Using mouse embryo fibroblast from these animals, however, we showed that p21 is required for the G1 DNA damage checkpoint activated by p53. Other p53 dependent functions such as apoptosis are unaffected in these cells. For Aim 5, we have performed a number of studies (some of which are collaborative with other groups) that have characterized the interactions of p21 with Cdks (52), examined is biochemical function in the G1 DNA damage checkpoint (53, 54), and examined the effects of p21 on PCNA dependent DNA replication in vitro (55). p21 is associated with PCNA in cells, although the significance of this is

currently unknown. In addition, we have identified a p21 homolog - p57 - and characterized its interaction with Cdks (46).

Table 1: Summary of Our Published Data

Aim	Major Original Objectives	Publications
1	determine expression patterns of p21 during development and in adult; determine p53 dependence	*Science* 267, 1024-1027 (1995)
2	construct p21 deficient ES cells by homologous recombination; use cells to generate chimaeric mouse and breed to homozygosity	*Cell* 82, 675-684 (1995)
3	characterize phenotype of p21 deficient mouse, including developmental effects, tumorigenesis, effects of p21 loss on DNA damage checkpoint control; Collaboration of p21-deficiency And mutations in other CKIs	*Cell* 82, 675-684 (1995) *Genes Dev.* in press (1998) see this progress report
4	Analysis of p21 expression in breast tumors and cell lines	see 1997 progress report
5	Identification of regulators of p21, analysis of targets of p21 including p21 associated proteins, regulation by phosphorylation, regulation of p21 expression by DNA damage, effects of loss of p57 on p21 levels	*Mol. Biol. Cell* 6, 387-400 (1995) *PNAS* 91, 8655-8659 (1994) *Cancer Res.* 54, 1169-1174 (1994) *Cell*, 76, 1013-1023 (1994) *Nature*, 387, 151-158 (1997) *Genes Dev* 9, 650-662 (1995) *Meth Enz.* 283, 230-244 (1997) see 1997 progress report
---	reviews emanating from this project	*Trends in Cell Biology* 6, 388-392 (1996) *Cancer Surveys* 29, 91-108 (1997) *Curr Opin Cell Biology*, 6, 847-852 (1994) *Curr Opin Gen Devel* 6, 56-64 (1996) *Science* 274, 1664-1672 (1996)

The major goal of Aim 4 was to examine p21 expression in mammary cells and to explore the question of whether p53 regulates p21 levels in these cells. As detailed in the 1997 progress report, we have addressed this question in part through an analysis of p21 expression in normal and tumor mammary epithelial cells and therefore will not be detailed here. In summary, we collaborated with Brian Dynlacht to generate a panel of monoclonal antibodies against p21 (59) as well as polyclonal antibodies and used these to study expression of p21 in normal and tumor breast epithelial cells, the latter experiments being done with Khandan Keyomarsi. The results indicated that p21 levels are cell cycle regulated and in some but not all tumor mammary tumor cells, p21 levels are reduced. The

significance of these observations are unknown and we have attempted to publish them. A large number of studies have looked for mutations in the p21 gene but these efforts have only rarely yielded data consistent with mutations of p21 in cancer. Because of the large number of studies that have been performed, we have not pursued in great detail the question of whether p21 is mutated in human breast cancer. The overwhelming mass of data indicates that it is not but that the related inhibitor p27 may display altered regulation in breast tumors.

Extension of Specific Aims 2 and 3.

Our major goal in this work has been to understand the role of p21 in development and transformation. The finding by our lab and other labs that p21 is a member of a protein family containing p27 and p57 was unforeseen at the time the initial grant was written in 1993. Studies during this period have revealed that there is likely to be extensive functional overlap between different members of the CKI family. This is amplified by the extensive cell type specificity observed with the expression of various CKIs.

These question of functional redundancy is raised most profoundly by studies of knockout mice where one particular CKI may fulfill the function of the CKI lost by directed deletion of the gene. For example, since p21 knockout mice have no obvious developmental phenotype, it was possible that other CKIs (p27 or p57) were up-regulated in response to p21 loss and that they perform any essential functions of p21 in vivo. However, in our previous study, we were unable to find any alterations in the expression of p27 or p57 in p21 knockout mice (51). In a separate study, we have generated mice lacking p57. We examined whether mice lacking p57 compensated for its loss by inducing either p21 or p57. We found no change in the levels of p21 in either muscle or kidney by immunoblotting. While p27 levels were also unaltered in kidney, there was a slight (50%) increase in p27 levels in p57 -deficient mice (see Figure 1 in Zhang et al., in the appendix).

In order to examine the question of redundancy in greater detail, we cross p21-/- mice to both p27 and p57-deficient mice to create double knockout mice. The expectation is that specific tissues which employ redundant utilization of multiple inhibitors might give rise to more severe phenotypes than the single mutants alone. In a parallel series of studies we have found that p27 and p57 collaborate to control cell cycle exit in the differentiating lens (58). Thus, there are clear cases where CKIs act redundantly. As described below, we now have evidence that p21 and p57 also act redundantly to control development in the lungs. In contrast, we have yet to observe any combinatorial phenotypes in the p21/p27 double knockout mice.

Generation of mice lacking both $p21^{CIP1}$ and $p57^{KIP2}$. To generate mice lacking p21 and p57, we crossed $p21^{+/-}$ $p57^{-p/+}$ females to $p21^{-/-}$ males. Animals inheriting the mutant p57 allele from the mother have a p57 null phenotype because imprinting renders the paternally-inherited allele silent. Consistent with our previous report (Zhang et al, 1997), there were no live born mice lacking either p57 or both p21 and p57 functions. However, E16.5 embryos of all genotypes were detected at Mendelian ratios. A substantial fraction of $p57^{-m/+}$ single mutant (30%) and $p21^{-/-}$ $p57^{-m/+}$ double mutant (65%) embryos die in utero due to placental failure (Table 1). Thus, loss of p21 exacerbates the placental defects observed in $p57^{-m/+}$ mutants. The following phenotypic analysis on $p21^{-/-}$ $p57^{-m/+}$ double mutants was based on animals that were not affected by placental failures.

$p21^{-/-}p57^{-m/+}$ double mutants show altered lung development. Histopathological examination of $p21^{-/-}p57^{-m/+}$ mice revealed all of the phenotypes caused

by $p57^{-p/+}$ loss alone (57) and novel phenotypes in tissues that are apparently unaffected in either of the single mutant animals. Unlike $p21^{-/-}$ or

Table 1. Distribution of genotypes among embryos derived from a cross between p21-/- p57+/+ males and p21+/- p57+/-p females.

Genotype	p21+/- p57+/+	p21-/- p57+/+	p21+/- p57+/-m	p21-/- p57+/-m
# of embryos	26	24	20 (6)[a]	29(19)[a]
Observed (%)	26	24	20	29
Expected (%)	25	25	25	25
Lethality (%)	0	0	30	65

[a] Number in parentheses indicates the number of embryos that were already dead at the time of harvesting.

$p57^{+/-m}$ animals, the lungs of $p21^{-/-}p57^{-m/+}$ animals were clearly defective, failing to fully differentiate distal air sacs, the ultimate functioning unit for gas exchange in lung tissue. The mammalian lung is composed of two types of tissues, an epithelium that lines all the airways from the trachea to alveoli and a mesenchymal stroma that supports the epithelium. Lung development is divided into several periods. In the pseudoglandular period early during embryogenesis, the lung resembles an exocrine gland and consists of a complex of branching bronchial tubes that include the primary, secondary, segmental and terminal bronchi, and the bronchioles. This is followed by the canalicular period when respiratory bronchioles are formed. Each respiratory bronchiole is terminated in two or three thin-walled dilations termed terminal sacs or primitive alveoli. At E16.5, lungs from WT embryos display substantial formation of primitive alveoli manifested as "open spaces" on H&E stained sections (Figure 1A, a). In contrast, lungs from $p21^{-/-}p57^{+/-m}$ animals are virtually devoid of "open spaces" (Figure 1A, c). Under high magnification, it is evident that primitive alveoli do not develop in the double mutants (Figure 1 A, compare d and e). This defect persists until birth (Figure 1A, compare f and g). Furthermore, there is a decrease in the size of the lumenal space of the bronchi and bronchioles in the double mutants. $p21^{+/-}$ $p57^{+/-m}$ lungs exhibit an intermediate phenotype between the WT and the double mutant with some primitive alveoli but fewer than in the WT (Figure 1A, compare a, b and c), indicating a single p21 gene is insufficient in the absence of p57.

To explore the cause of the lung defect, we examined the expression of both p57 and p21 in the developing lung. p57 is highly expressed in bronchiole epithelium mirroring that of CC10, a marker for that tissue. p57 is expressed at lower levels in an undefined subset of lung mesenchymal cells and the epithelium lining of the terminal primitive alveoli (Figure 1B). In contrast, p21 is expressed throughout the lung (Figure 1). Despite high levels of

expression of p57 in the bronchiole epithelium, no significant abnormalities were detected in this tissue and tissue specific differentiation markers such as CC10 and SP-A, B and C are expressed normally in the double mutants (Figure 1). Although the absence of air sac lumenal space gives the appearance of increased cellularity in the mutants, this is not the case. This is due to the fact that the lungs of the double mutant mice are smaller than the wild-type lungs, thus the total number of cells are approximately the same. Furthermore, the overall proliferation rates in the double mutant lung were not elevated as judged by BrdU pulse labeling nor was there an increase in apoptosis. Thus, the defects in primitive alveoli formation in the absence of p21 and p57 is likely to result from subtle changes in the differentiation of either the epithelia or the mesenchymal stroma for which additional studies are required to delineate more precisely.

Skeleton defects in $p21^{-/-}$ $p57^{-m/+}$ double mutants. The only phenotype of $p57^{+/-m}$ mice that is enhanced by loss of p21 is the skeletal phenotype. Deletion of p57 alone causes delay in ossification and sternal fusion defects, but no overall abnormality in the shape of the skeleton (57). However, as shown in Figure 2, $p21^{-/-}$ $p57^{-m/+}$ double mutant embryos display a posture clearly distinct from those of WT and $p57^{-m/+}$ mutants (Figure 2A-C). Skeleton staining revealed that double mutants (Figure 2E) lack the spinal curvature seen in WT (Figure 2D) and $p57^{-m/+}$ single mutants (data not shown), which might stem from defects in musculature (see below). Rib cage shape in double mutant embryos is also abnormal (Figure 2, compare D and E). Bifurcation of ribs was observed in double mutants, usually of the 9th rib (Figure 2F) although occasionally the 7th rib is also affected (Figure 2J). The femurs of double mutant lack a cartilage outgrowth seen in either p21 or p57 single mutants or WT littermates (Figure 2G and data not shown). The double mutants exhibited sternum fusion defects similar to those seen in p57 single mutants (Zhang, et al, 1997), but the sternum of double mutants is shorter than that of p57 single mutants (Figure 2H). The ribs of double mutants join the sternum at an angle of 90°(Figure 2J), while the ribs of WT or p57 single mutants join at an angle much less than 90° (Figure 2H). Both p21 and p57 have been found highly expressed in developing ribs (Zhang et al, 1997, and data not shown). However, it is difficult to distinguish autonomous vs. nonautonomous roles of these two inhibitors in ribs, especially considering the fact that similar defects in the attachment of ribs to sternum are observed in mice lacking myogenin (59).

We are still in the process of analyzing various phenotypes in the p21/p27 double knockout animals but at this point we already have evidence of collaboration. We envision further analysis of these mice will provide findings that we will submit for publication.

Conclusion:

Proliferation control is vital to a developing organism. The cyclin-dependent kinase inhibitors (CKIs) are good candidates for molecules providing the controls on cellular proliferation required for embryonic developmental programs because of their biochemical properties and their patterns of expression. For example, we have shown that p21 is highly expressed in a number of terminally differentiating tissues (49). Surprisingly, however, mice lacking either p16, p21, or p27 display normal embryonic development (60-62), suggesting that other cell cycle regulatory mechanisms might exist to compensate for their loss. The present work reveals such a redundancy and demonstrates that two CKIs, p21 and p57, cooperate to control proliferation and differentiation in the lungs.

The lung develops through interactions between an epithelial tissue that lines airways and a mesenchymal tissue that surrounds the epithelium. It is likely that in the absence of p21 and p57, the epithelium-mesenchyme interactions are somehow disrupted in the

formation of primitive alveoli. This disruption is stage specific since development of the bronchial tree is unaffected. Thus far we have been unable to more precisely define the cell types defective in the lung of mutant animals because p21 and p57 were co-expressed in a number of different cell types and no morphological differences are observed among them.

The lung defects appear to be quite distinct from those observed in the lens lacking both p27 and p57 (58). First, it is likely that novel signal transduction pathways are used to control p21 and p57 in lung differentiation pathways. Second, unlike the lens, the defects in the formation of primitive alveoli do not appear to be a result of overproliferation or apoptosis. It is possible that these two CKIs contribute directly to the differentiation of either the epithelial or the mesenchymal cells in a cell cycle-independent way. Alternatively, the increased Cdk activity caused by lack of the inhibitors might be insufficient to drive the G1/S transition but sufficient to interfere with the differentiation processes. Analysis of Rb mutants has shown that it is possible to separate the cell cycle and differentiation functions of Rb (63), although whether it is possible to separate these functions by differential Cdk-dependent phosphorylation is not known. Alternatively, there could exist Cdk-dependent pathways parallel to Rb that are required for differentiation. In support of this, Skapek et al. (64) have shown that additional cyclin D1 could block myogenesis even in the presence of non-phosphorylatable and presumably constitutively active Rb. Clearly, however, different tissues can be dependent upon the same two regulators in different ways, underscoring the complex relationship between the cell cycle and development.

The last two years has seen a virtual explosion in our understanding of the mechanisms regulating cell cycle progression. Much of this concerns the role of Cdk inhibitors in cell cycle control. Our work funded under this grant involves an analysis of the role of p21 through generation of p21-knockout mice and through biochemical analysis of p21 function. Our approach has been to address patterns of expression during development, to determine whether p21 is required for the p53 checkpoint, tumor suppression, and or development through analysis of mice deficient in p21, and to understand mechanistic aspects of p21 function within the context of other CKIs including p57. Our major contribution has been to demonstrate that p21 is not required for the tumor suppression function of p53 at least in the mouse but that it is involved in G1 checkpoint control. This is an important finding since it focuses mechanistic studies on p53 to other pathways that may be important for tumor suppression such as apoptosis.

There are a number of outstanding questions related to p21 function and regulation which we will continue to pursue in the future. In particular, we would like to understand the consequences of loss of multiple inhibitors of regulation of the cell cycle machinery and S-phase entry. The prediction is that the Rb pathway will be inappropriately activated in double knockout cells where two inhibitors act redundantly. This will need to be examined experimentally. In addition, we will need to understand the contribution, if any, of CKIs to differentiation independent of their cell cycle arrest function. There is evidence from the p57 knockout that this inhibitor functions in differentiation and the lung phenotype described above may also have aspects of uncoordinated differentiation. This will be pursued in the future. Overall, our studies on p21 have provided important new information for the G1 damage checkpoint, cell cycle regulation and redundancy among CKIs.

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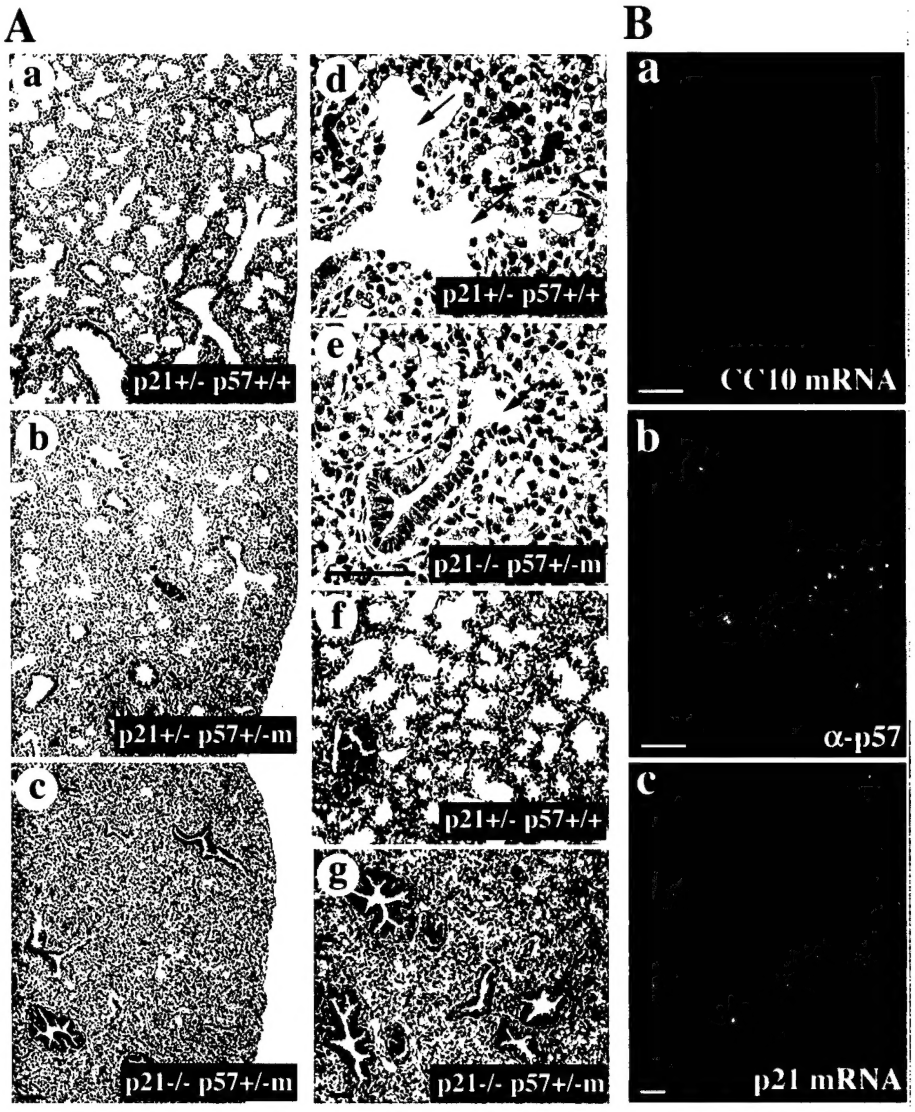
Figure Legends

Figure 1. A block in the formation of primitive alveoli in the absence of both p21 and p57. (A) Hematoxylin and eosin-stained transverse sections of lungs derived from E16.5 (a, b, c , d and e) and of E18.5 embryos (f and g). (B) Expression of p21 and p57 in the lung of E18.5 embryos. (a) CC10 expression detected with in situ hybridization. (b) Immunofluorescence staining of p57. (c) p21 expression detected with in situ hybridization. Scale bars, 200 μm.

Figure 2. Skeletal defects in $p21^{-/-}p57^{m/+}$ mutants. (A, B, C) $p21^{-/-}p57^{m/+}$ embryos display altered posture. (D, E) Skeletons of E18.5 embryos stained with alcian blue to identify cartilage and alizarin red to identify ossified bone. (F) Bifurcation of the 9th rib (arrow) is observed in $p21^{-/-}p57^{m/+}$ embryos. (G) The femur of E18.5 embryos stained with alcian blue and alizarin red. Arrow indicates the cartilage outgrowth. (H, I, J) Sternia and ribs of E18.5 embryos stained with alcian blue and alizarin red. Only 7 of the 13 ribs attach to the sternum. Note a bifurcation in the 7th rib in the double mutant (arrow in J).


A
B
a
p21+/- p57+/+
b
p21+/- p57+/-m
c
p21-/- p57+/-m
d
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e
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f
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g
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CC10 mRNA
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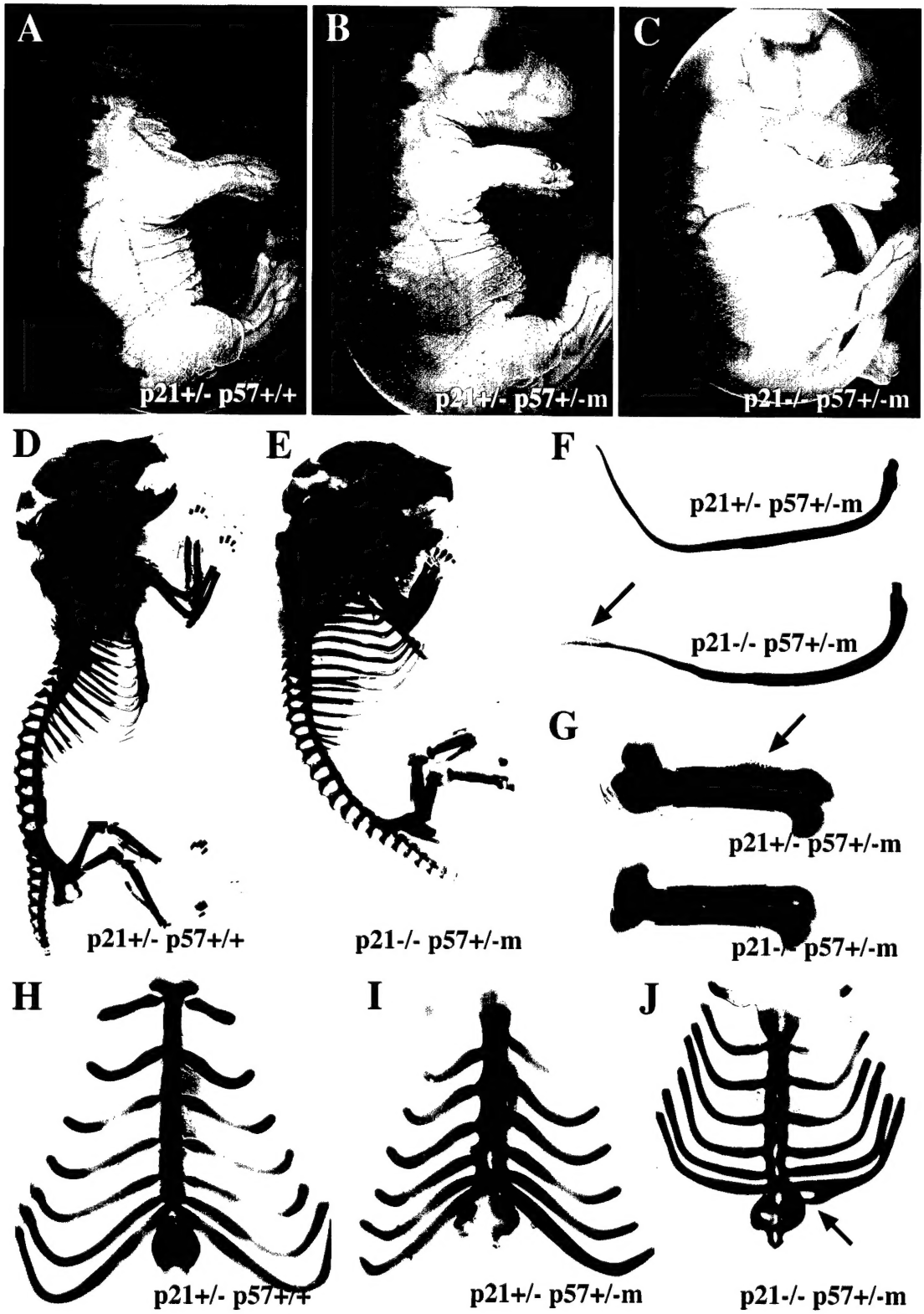
α-p57
c
p21 mRNA

Figure 1


A
p21+/- p57+/+
B
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C
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E
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H
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I
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J
p21-/- p57+/-m

Figure 2

Personnel
J. Wade Harper (P.I.)
Stephen J. Elledge (Co-P.I.)
Jeff Winston
Chang Bai
Pumin Zhang

Collaborators
Phil Leder
Steve Reed
Bert Vogelstein
Khandan Keyomarsi
Ed Harlow
Brian Dynlacht

Publications

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